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(54) Title: HEAT STABLE MUTANTS OF STARCH BIOSYNTHESIS ENZYMES

(57) Abstract: The subject invention pertains to novel mutant polynucleotide molecules that encode enzymes that have increased heat stability. These polynucleotides, when expressed in plants, result in increased yield in plants grown under conditions of heat stress. In one embodiment, the polynucleotide molecules of the subject invention encode maize endosperm ADP glucose pyrophosphorylase (AGP) and soluble starch synthase (SSS) enzyme activities. Plants and plant issue bred to contain, or transformed with, the mutant polynecleotides, and expressing the polypeptides encoded by the polynucleotides, are also contemplated by the present invention. The subject invention also concerns methods for isolating polynucleotides and polypeptides contemplated within the scope of the invention. Methods for increasing yield in plants grown under conditions of heat stress are also provided.

DESCRIPTION

HEAT STABLE MUTANTS OF STARCH BIOSYNTHESIS ENZYMES

This invention was made with government support under National Science Foundation grant number 9316887. The government has certain rights in the invention.

Cross-Reference to Related Application

This application claims the benefit of U.S. Provisional application Serial No. 60/275,768, filed March 14, 2001.

Background of the Invention

The sessile nature of plant life generates a constant exposure to environmental factors that exert positive and negative effects on its growth and development. One of the major impediments facing modern agriculture is adverse environmental conditions. One important factor which causes significant crop loss is heat stress. Temperature stress greatly reduces grain yield in many cereal crops such as maize, wheat, and barley. Yield decreases due to heat stress range from 7 to 35% in the cereals of world-wide importance.

A number of studies have identified likely physiological consequences of heat stress. Early work by Hunter *et al.* (Hunter, R. B., Tollenaar, M., and Breuer, C. M. [1977] *Can. J. Plant Sci.* 57:1127-1133) using growth chamber conditions showed that temperature decreased the duration of grain filling in maize. Similar results in which the duration of grain filling was adversely altered by increased temperatures were identified by Tollenaar and Bruulsema (Tollenaar, M. and Bruulsema, T. W. [1988] *Can. J. Plant Sci.* 68:935-940). Badu-Apraku *et al.* (Badu-Apraku, B., Hunter, R. B., and Tollenaar, M. [1983] *Can. J. Plant. Sci.* 63:357-363) measured a marked reduction in the yield of maize plants grown under the day/night temperature regime of 35/15°C compared to growth in a 25/15°C temperature regime. Reduced yields due to increased temperatures is also supported by historical as well as climatological studies (Thompson, L. M. [1986] *Agron. J.* 78:649-653; Thompson, L. M. [1975] *Science* 188:535-541; Chang, J. [1981]

Agricul. Metero. 24:253-262; and Conroy, J. P., Seneweera, S., Basra, A. S., Rogers, G., and Nissen-Wooller, B. [1994] *Aust. J. Plant Physiol.* 21:741-758).

That the physiological processes of the developing seed are adversely affected by heat stress is evident from studies using an *in vitro* kernel culture system (Jones, R.J., Gengenbach, B.G., and Cardwell, V.B. [1981] *Crop Science* 21:761-766; Jones, R.J., Ouattar, S., and Crookston, R.K. [1984] *Crop Science* 24:133-137; and Cheikh, N., and Jones, R.J. [995] *Physiol. Plant.* 95:59-66). Maize kernels cultured at the above-optimum temperature of 35°C exhibited a dramatic reduction in weight.

Work with wheat identified the loss of soluble starch synthase (SSS) activity as a hallmark of the wheat endosperm's response to heat stress (Hawker, J. S. and Jenner, C. F. [1993] *Aust. J. Plant Physiol.* 20:197-209; Denyer, K., Hylton, C. M., and Smith, A. M. [1994] *Aust. J. Plant Physiol.* 21:783-789; Jenner, C. F. [1994] *Aust. J. Plant Physiol.* 21:791-806). Additional studies with SSS of wheat endosperm show that it is heat labile (Rijven, A.H.G.C. [1986] *Plant Physiol.* 81:448-453; Keeling, P.L., Bacon, P.J., Holt, D.C. [1993] *Planta.* 191:342-348; Jenner, C. F., Denyer, K., and Guerin, J. [1995] *Aust. J. Plant Physiol.* 22:703-709).

The roles of SSS and ADP glucose pyrophosphorylase (AGP) under heat stress conditions in maize is less clear. AGP catalyzes the conversion of ATP and α-glucose-1-phosphate to ADP-glucose and pyrophosphate. ADP-glucose is used as a glycosyl donor in starch biosynthesis by plants and in glycogen biosynthesis by bacteria. The importance of ADP-glucose pyrophosphorylase as a key enzyme in the regulation of starch biosynthesis was noted in the study of starch deficient mutants of maize (*Zea mays*) endosperm (Tsai, C.Y., and Nelson, Jr., O.E. [1966] *Science* 151:341-343; Dickinson, D.B., J. Preiss [1969] *Plant Physiol.* 44:1058-1062).

Ou-Lee and Setter (Ou-Lee, T. and Setter, T.L. [1985] *Plant Physiol.* 79:852-855) examined the effects of temperature on the apical or tip regions of maize ears. With elevated temperatures, AGP activity was lower in apical kernels when compared to basal kernels during the time of intense starch deposition. In contrast, in kernels developed at normal temperatures, AGP activity was similar in apical and basal kernels during this period. However, starch synthase activity during this period was not differentially affected in apical and basal kernels. Further, heat-treated apical kernels exhibited an increase in starch synthase activity over control. This was not observed with AGP activity.

Singletary *et al.* (Singletary, G.W., Banisadr, R., and Keeling, P.L. [1993] *Plant Physiol.* 102: 6 (suppl).; Singletary, G.W., Banisadra, R., Keeling, P.L. [1994] *Aust. J. Plant Physiol.* 21:829-841) using an *in vitro* culture system quantified the effect of various temperatures during the grain fill period. Seed weight decreased steadily as temperature increased from 22-36°C. A role for AGP in yield loss is also supported by work from Duke and Doehlert (Duke, E.R. and Doehlert, D.C. [1996] *Environ. Exp. Botany.* 36:199-208).

Work by Keeling *et al.* (1994, *supra*) quantified SSS activity in maize and wheat using Q_{10} analysis, and showed that SSS is an important control point in the flux of carbon into starch.

In vitro biochemical studies with AGP and SSS clearly show that both enzymes are heat labile. Maize endosperm AGP loses 96% of its activity when heated at 57°C for five minutes (Hannah, L.C., Tuschall, D.M., and Mans, R.J. [1980] *Genetics* 95:961-970). This is in contrast to potato AGP which is fully stable at 70°C (Sowokinos, J.R. and Preiss, J. [1982] *Plant Physiol.* 69:1459-1466; Okita, T.W., Nakata, P.A., Anderson, J.M., Sowokinos, J., Morell, J., and Preiss, J. [1990] *Plant Physiol.* 93:785-90). Heat inactivation studies with SSS showed that it is also labile at higher temperatures, and kinetic studies determined that the K*m* value for amylopectin rose exponentially when temperature increased from 25-45°C (Jenner *et al.*, 1995, *supra*).

Biochemical and genetic evidence has identified AGP as a key enzyme in starch biosynthesis in higher plants and glycogen biosynthesis in *E. coli* (Preiss, J. and Romeo, T. [1994] *Progress in Nuc. Acid Res. and Mol Biol.* 47:299-329; Preiss, J. and Sivak, M. [1996] "Starch synthesis in sinks and sources," In *Photoassimilate distribution in plants and crops: source-sink relationships*. Zamski, E., ed., Marcil Dekker Inc. pp. 139-168). AGP catalyzes what is viewed as the initial step in the starch biosynthetic pathway with the product of the reaction being the activated glucosyl donor, ADPglucose. This is utilized by starch synthase for extension of the polysaccharide polymer (reviewed in Hannah, L. Curtis [1996] "Starch synthesis in the maize endosperm," In: *Advances in Cellular and Molecular Biology of Plants*, Vol. 4. B. A. Larkins and I. K. Vasil (eds.). Cellular and Molecular Biology of Plant Seed Development. Kluwer Academic Publishers, Dordrecht, The Netherlands).

Initial studies with potato AGP showed that expression in *E. coli* yielded an enzyme with allosteric and kinetic properties very similar to the native tuber enzyme (Iglesias, A., Barry, G.F., Meyer, C., Bloksberg, L., Nakata, P., Greene, T., Laughlin, M.J., Okita, T.W., Kishore, G.M., and Preiss, J. [1993] *J. Biol Chem.* 268:1081-86; Ballicora, M.A., Laughlin, M.J., Fu, Y., Okita, T.W., Barry, G.F., and Preiss, J. [1995] *Plant Physiol.* 109:245-251). Greene *et al.* (Greene, T.W., Chantler, S.E., Kahn, M.L., Barry, G.F., Preiss, J., and Okita, T.W. [1996] *Proc. Natl. Acad. Sci.* 93:1509-1513; Greene, T.W., Woodbury, R.L., and Okita, T.W. [1996] *Plant Physiol.* (112:1315-1320) showed the usefulness of the bacterial expression system in their structure-function studies with the potato AGP. Multiple mutations important in mapping allosteric and substrate binding sites were identified (Okita, T.W., Greene, T.W., Laughlin, M.J., Salamone, P., Woodbury, R., Choi, S., Ito, H., Kavakli, H., and Stephens, K. [1996] "Engineering Plant Starches by the Generation of Modified Plant Biosynthetic Enzymes," In *Engineering Crops for Industrial End Uses*, Shewry, P.R., Napier, J.A., and Davis, P., eds., Portland Press Ltd., London).

AGP enzymes have been isolated from both bacteria and plants. Bacterial AGP consists of a homotetramer, while plant AGP from photosynthetic and non-photosynthetic tissues is a heterotetramer composed of two different subunits. The plant enzyme is encoded by two different genes, with one subunit being larger than the other. This feature has been noted in a number of plants. The AGP subunits in spinach leaf have molecular weights of 54 kDa and 51 kDa, as estimated by SDS-PAGE. Both subunits are immunoreactive with antibody raised against purified AGP from spinach leaves (Copeland, L., J. Preiss (1981) *Plant Physiol.* 68:996-1001; Morell, M., M. Bloon, V. Knowles, J. Preiss [1988] *J. Bio. Chem.* 263:633). Immunological analysis using antiserum prepared against the small and large subunits of spinach leaf showed that potato tuber AGP is also encoded by two genes (Okita *et al.*, 1990, *supra*). The cDNA clones of the two subunits of potato tuber (50 and 51 kDa) have also been isolated and sequenced (Muller-Rober, B.T., J. Kossmann, L.C. Hannah, L. Willmitzer, U. Sounewald [1990] *Mol. Gen. Genet.* 224:136-146; Nakata, P.A., T.W. Greene, J.M. Anderson, B.J. Smith-White, T.W. Okita, J. Preiss [1991] *Plant Mol. Biol.* 17:1089-1093). The large subunit of potato tuber AGP is heat stable (Nakata *et al.* [1991], *supra*).

As Hannah and Nelson (Hannah, L.C., O.E. Nelson (1975) *Plant Physiol.* 55:297-302.; Hannah, L.C., and Nelson, Jr., O.E. [1976] *Biochem. Genet.* 14:547-560) postulated, both *Shrunken-2* (*Sh2*) (Bhave, M.R., S. Lawrence, C. Barton, L.C. Hannah [1990] *Plant Cell* 2:581-588) and *Brittle-2* (*Bt2*) (Bae, J.M., M. Giroux, L.C. Hannah [1990] *Maydica* 35:317-322) are structural genes of maize endosperm ADP-glucose pyrophosphorylase. *Sh2* and *Bt2* encode the large subunit and small subunit of the enzyme, respectively. From cDNA sequencing, *Sh2* and *Bt2* proteins have predicted molecular weight of 57,179 Da (Shaw, J.R., L.C. Hannah [1992] *Plant Physiol.* 98:1214-1216) and 52,224 Da, respectively. The endosperm is the site of most starch deposition during kernel development in maize. *Sh2* and *bt2* maize endosperm mutants have greatly reduced starch levels corresponding to deficient levels of AGP activity. Mutations of either gene have been shown to reduce AGP activity by about 95% (Tsai and Nelson, 1966, *supra*; Dickinson and Preiss, 1969, *supra*). Furthermore, it has been observed that enzymatic activities increase with the dosage of functional wild type *Sh2* and *Bt2* alleles, whereas mutant enzymes have altered kinetic properties. AGP is the rate limiting step in starch biosynthesis in plants. Stark *et al.* placed a mutant form of *E. coli* AGP in potato tuber and obtained a 35% increase in starch content (Stark *et al.* [1992] *Science* 258:287).

The cloning and characterization of the genes encoding the AGP enzyme subunits have been reported for various plants. These include *Sh2* cDNA (Bhave *et al.*, 1990, *supra*), *Sh2* genomic DNA (Shaw and Hannah, 1992, *supra*), and *Bt2* cDNA (Bae *et al.*, 1990, *supra*) from maize; small subunit cDNA (Anderson, J.M., J. Hnilo, R. Larson, T.W. Okita, M. Morell, J. Preiss [1989] *J. Biol. Chem.* 264:12238-12242) and genomic DNA (Anderson, J.M., R. Larson, D. Landencia, W.T. Kim, D. Morrow, T.W. Okita, J. Preiss [1991] *Gene* 97:199-205) from rice; and small and large subunit cDNAs from spinach leaf (Morell *et al.*, 1988, *supra*) and potato tuber (Muller-Rober *et al.*, 1990, *supra*; Nakata, P.A., Greene, T.W., Anderson, J.W., Smith-White, B.J., Okita, T.W., and Preiss, J. [1991] *Plant Mol. Biol.* 17:1089-1093). In addition, cDNA clones have been isolated from wheat endosperm and leaf tissue (Olive, M.R., R.J. Ellis, W.W. Schuch [1989] *Plant Physiol. Mol. Biol.* 12:525-538) and *Arabidopsis thaliana* leaf (Lin, T., Caspar, T., Sommerville, C.R., and Preiss, J. [1988] *Plant Physiol.* 88:1175-1181). AGP sequences from barley have also been described in Ainsworth *et al.* (Ainsworth, C., Hosein, F., Tarvis, M., Weir, F., Burrell, M., Devos, K.M., Gale, M.D. [1995] *Planta* 197:1-10).

AGP functions as an allosteric enzyme in all tissues and organisms investigated to date. The allosteric properties of AGP were first shown to be important in *E. coli.* A glycogen-overproducing *E. coli* mutant was isolated and the mutation mapped to the structural gene for AGP, designated as *gly*C. The mutant *E. coli*, known as *gly*C-16, was shown to be more sensitive to the activator, fructose 1,6 bisphosphate, and less sensitive to the inhibitor, cAMP (Preiss, J. [1984] *Ann. Rev. Microbiol.* 419-458). Although plant AGP's are also allosteric, they respond to different effector molecules than bacterial AGP's. In plants, 3-phosphoglyceric acid (3-PGA) functions as an activator while phosphate (PO_4) serves as an inhibitor (Dickinson and Preiss, 1969, *supra*).

Using an *in vivo* mutagenesis system created by the *Ac*-mediated excision of a *Ds* transposable element fortuitously located close to a known activator binding site, Giroux *et al.* (Giroux, M.J., Shaw, J., Barry, G., Cobb, G.B., Greene, T., Okita, T.W., and Hannah, L. C. [1996] *Proc. Natl. Acad. Sci.* 93:5824-5829) were able to generate site-specific mutants in a functionally important region of maize endosperm AGP. One mutant, *Rev6,* contained a tyrosine-serine insert in the large subunit of AGP and conditioned a 11-18% increase in seed weight. In addition, published international application WO 01/64928 teaches that various characteristics, such as seed number, plant biomass, Harvest Index *etc.*, can be increased in plants transformed with a polynucleotide encoding a large subunit of maize AGP containing the *Rev6* mutation.

Published international patent applications WO 99/58698 and WO 98/22601 and issued U.S. Patent No. 6,069,300 disclose mutations in the large subunit of maize AGP enzyme that, when expressed, confers increased heat stability in comparison to that observed for wild type AGP enzyme. Several heat stable mutants are disclosed in the '300 patent and WO publications, including mutants designated as HS 13 (having an Ala to Pro substitution at position 177); HS 14 (having an Asp to His substitution at position 400 and a Val to Ile substitution at position 454; HS 16 (having an Arg to Thr substitution at position 104); HS 33 (having a His to Tyr substitution at position 333); HS 39 (having a His to Tyr substitution at position 333); HS 40 (having a His to Tyr substitution at position 333 and a Thr to Ile substitution at position 460); HS 47 (having an Arg to Pro substitution at position 216 and a His to Tyr substitution at position 333); RTS 48-2 (having an Ala to Val substitution at position 177); and RTS 60-1 (having an Ala to Val substitution at position 396).

Brief Summary of the Invention

The subject invention pertains to materials and methods useful for improving crop yields in plants, such as those plants that produce cereal crops. In one embodiment, the subject invention provides heat stable AGP enzymes and nucleotide sequences which encode these enzymes. In a preferred embodiment, the heat stable enzymes of the invention can be used to provide plants having greater tolerance to higher temperatures, thus enhancing the crop yields from these plants. In a particularly preferred embodiment, the improved plant is a cereal. Cereals to which this invention applies include, for example, maize, wheat, rice, and barley.

Brief Description of the Drawings

Figure 1 shows the genomic nucleotide sequence of a wild type Shrunken-2 allele of *Zea mays*. Introns are indicated by lower case letters. Base number 1 is the transcription start site.

Figure 2 shows a comparison of enzyme activity for wild type and various maize AGP large subunit mutants. All reactions were performed in duplicate. Numbers given are the average of the duplicates, after background removal. The percentages refer to activity remaining after heat treatment as compared to activity prior to heat treatment. The legend for the figure is as follows:

"sh2" = wild type sh2 protein;

"sh2ht" = wild type sh2 protein, following heat treatment;

"33" = sh2 protein containing the *HS 33* mutation (*i.e.*, a histidine-to-tyrosine amino acid substitution at position 333 in the large subunit of maize AGP);

"33ht" = sh2 protein containing the *HS 33* mutation, following heat treatment;

"177" = sh2 protein containing the mutation *rts48-2* (*i.e.*, an alanine-to-valine amino acid substitution at position 177 in the large subunit of maize AGP);

"177ht" = sh2 protein containing the mutation *rts48-2* (*i.e.*, an alanine-to-valine amino acid substitution at position 177 in the large subunit of maize AGP), following heat treatment;

"396" = sh2 protein containing the mutation *rts60-1* (*i.e.*, an alanine-to-valine amino acid substitution at position 396 in the large subunit of maize AGP);

"396ht" = sh2 protein containing the mutation *rts60-1* (*i.e.*, an alanine-to-valine amino acid substitution at position 396 in the large subunit of maize AGP), following heat treatment;

"7+6" = sh2 protein containing the combination of "177" and "396"mutations;

"7+6ht" = sh2 protein containing the combination of "177" and "396"mutations, following heat treatment;

"7+3" = sh2 protein containing the combination of "177" and "HS 33"mutations;

"7+3ht"= sh2 protein containing the combination of "177" and "HS 33" mutations, following heat treatment;

"6+3" = sh2 protein containing the combination of "396" and "HS 33"mutations;

"6+3ht" = sh2 protein containing the combination of "396" and "HS 33" mutations, following heat treatment.

Figure 3 shows a restriction map of *Sh2* coding region. Restriction enzymes shown are those used in isolation of entire coding region and in creation of double and triple mutants. Mutations are indicated with asterisks (*).

Brief Description of the Sequences

SEQ ID NO. 1 is an amino acid sequence of a region corresponding to amino acids 318 to 350 of the large subunit of AGP in maize containing the HS 33 mutation.

SEQ ID NO. 2 is an amino acid sequence of a region corresponding to amino acids 170 to 189 of the large subunit of AGP in maize containing the RTS48-2 mutation.

SEQ ID NO. 3 is an amino acid sequence of a region corresponding to amino acids 389 to 406 of the large subunit of AGP in maize containing the RTS60-1 mutation.

SEQ ID NO. 4 is the genomic nucleotide sequence of a wild type Shrunken-2 allele of *Zea mays*.

SEQ ID NO. 5 is a synthetic oligonucleotide primer that can be used in accordance with the subject invention.

SEQ ID NO. 6 is a synthetic oligonucleotide primer that can be used in accordance with the subject invention.

SEQ ID NO. 7 is a synthetic oligonucleotide primer that can be used in accordance with the subject invention.

SEQ ID NO. 8 is a synthetic oligonucleotide primer that can be used in accordance with the subject invention.

SEQ ID NO. 9 is a synthetic oligonucleotide primer that can be used in accordance with the subject invention.

SEQ ID NO. 10 is a synthetic oligonucleotide primer that can be used in accordance with the subject invention.

Detailed Disclosure of the Invention

The subject invention concerns novel mutant polynucleotide molecules, and the polypeptides encoded thereby, that confer increased heat resistance and yield in plants grown under conditions of heat stress relative to plants expressing wild type genotype. In specific embodiments, the polynucleotide molecules of the subject invention encode maize endosperm ADP glucose pyrophosphorylase (AGP) and soluble starch synthase (SSS) enzyme activities. The mutant enzymes confer increased stability to heat stress conditions during seed and plant development in seeds and plant tissue expressing the enzymes as compared with wild type enzyme activities.

One aspect of the subject invention concerns polynucleotides which encode two or more amino acid changes in an AGP large subunit as compared to the wild type sequence of the AGP large subunit polypeptide, wherein the expressed mutant protein exhibits increased stability. Preferably, the polypeptide encoded by the subject polynucleotides, when expressed with the small subunit, exhibit increased enzymatic activity as compared to wild type protein and, preferably, at a level about the same or greater than that exhibited by a single amino acid mutation that confers increased heat stability, such as HS 33. The polynucleotides of the invention may encode two, three, or more amino acid changes from the wild type sequence. Preferably, a polynucleotide of the invention encodes a polypeptide having an amino acid substitution at one or more of the following positions corresponding to the position in the large subunit of maize AGP: position 177, 333, and 396.

In one embodiment, a polynucleotide of the present invention encodes a mutant large subunit of a plant AGP containing a double mutation: a histidine-to-tyrosine amino acid substitution and an alanine-to-valine amino acid substitution in the sequence of the polypeptide. In an exemplified embodiment, the histidine to tyrosine substitution occurs

at the amino acid corresponding to residue number 333 in the sequence of the large subunit of maize AGP. In one embodiment, the alanine-to-valine substitution occurs at the amino acid corresponding to residue number 177 in the sequence of the large subunit of maize AGP. In another embodiment, the alanine-to-valine substitution occurs at the amino acid corresponding to residue 396 in the sequence of the large subunit of maize AGP.

In a further embodiment, a polynucleotide of the present invention encodes a mutant large subunit of a plant AGP containing two alanine-to-valine amino acid substitutions within the sequence of the polypeptide. In an exemplified embodiment, the first alanine-to-valine substitution occurs at the amino acid corresponding to residue number 177 and the second alanine-to-valine substitution occurs at the amino acid corresponding to residue number 396 in the sequence of the large subunit of maize AGP. Enzyme activity associated with mutant proteins of the present invention having two mutations are shown in Figure 2.

Another embodiment concerns a triple mutant comprising a histidine to tyrosine substitution at the amino acid corresponding to residue number 333, an alanine-to-valine substitution at the amino acid corresponding to residue number 177, and an alanine-to-valine substitution at the amino acid corresponding to residue 396 in the sequence of the large subunit of maize AGP.

The amino acid residue numbers referred to above are based on the accepted number of the amino acids in this protein (Shaw and Hannah, 1992, *supra*). The position of these substitutions can be readily identified by a person skilled in the art. Table 1 below shows the double and triple amino acid substitution mutants exemplified herein.

Table 1.

Sh2 Polypeptide Mutant	Amino Acid Change
HS 7+3	Ala to Val at position 177 and His to Tyr at position 333
HS 6+3	Ala to Val at position 396 and His to Tyr at position 333
HS 7+6	Ala to Val at position 177 and Ala to Val at position 396
HS 7+6+3	Ala to Val at position 177 and Ala to Val at position 396 and His to Tyr at position 333

Because of the homology of AGP polypeptides between various species of plants (Smith-White and Preiss [1992] *J. Mol. Evol.* 34:449-464), the ordinarily skilled artisan can readily determine the position of the mutations in AGP from plants other than maize that correspond to the position of mutations in maize AGP as disclosed herein. Thus, the present invention encompasses polynucleotides that encode mutant AGP of plants other than maize, including, but not limited to, wheat, barley, oats, and rice, that confers increased heat stability when expressed in the plant.

Single amino acid mutations in AGP that confer heat stability, and methods for producing and selecting for such mutations, are disclosed in U.S. Patent No. 6,069,300 and published international applications WO 99/58698 and WO 98/22601. Typically, a plasmid comprising a polynucleotide coding for the SH2 subunit of maize AGP was mutagenized, placed into mutant *E. coli glg* C^- cells expressing the BT2 subunit, and the cells grown at 42°C to select for mutants that could produce glycogen at that temperature. Several mutants, termed heat stable (HS) mutants, were isolated. Crude extracts of these mutants were prepared and the heat stability of the resulting AGP was monitored. The single amino acid substitution mutants retained between 8-59% of their activity after incubation at 60°C for five minutes. In addition, total enzymatic activity of the mutant maize endosperm AGP before heat treatment was elevated about two- to three-fold in several of the mutants.

Multiple heat stability conferring mutations can easily be combined within one subunit. For example, different unique restriction sites that divide the coding regions of *Sh2* into three distinct fragments can be used. Where appropriate, mutation combinations can be generated by subcloning the corresponding fragment containing the added mutation. If two mutations are in close proximity, then site-directed mutagenesis can be used to engineer such combinations. One method for site specific mutations involves PCR, mutagenic primer, and the use of *Dpn*I restriction endonuclease. Primers can be constructed to contain the mutation in the 5′ end, and used to PCR amplify using the proofreading polymerase Vent. Amplified DNA can then be digested with *Dpn*I. Parental DNA isolated from *E. coli* is methylated and hence susceptible to *Dpn*I. Digested DNA is size fractionated by gel electrophoresis, ligated, and cloned into the expression vectors. Mutations are confirmed by sequence analysis and transformed into the AC70R1-504 strain carrying the wild type small subunit. Combinatorial mutants can then be analyzed.

The subject invention also concerns the mutant polypeptides, encoded by the subject polynucleotides, having the amino acid substitutions described herein. In a preferred embodiment, the mutant polypeptides are from maize.

The subject invention also concerns heat stable mutants of AGP of the present invention combined with heat stable mutations in the small subunit of the enzyme. Mutations in the small subunit of AGP that confer heat stability to the enzyme can also be readily prepared and identified using the methods described in U.S. Patent No. 6,069,300 and published international applications WO 99/58698 and WO 98/22601. Heat stable mutants of the small subunit can be co-expressed with the mutants of the present invention to further enhance the stability of an AGP enzyme.

Plants and plant tissue bred to contain or transformed with the mutant polynucleotides of the invention, and expressing the polypeptides encoded by the polynucleotides, are also contemplated by the present invention. Plants and plant tissue expressing the mutant polynucleotides produce tissues that have, for example, lower heat-induced loss in weight or yield when subjected to heat stress during development. Plants within the scope of the present invention include monocotyledonous plants, such as rice, wheat, barley, oats, sorghum, maize, lilies, and millet, and dicotyledonous plants, such as peas, alfalfa, chickpea, chicory, clover, kale, lentil, prairie grass, soybean, tobacco, potato, sweet potato, radish, cabbage, rape, apple trees, and lettuce. In a particularly preferred

embodiment, the plant is a cereal. Cereals to which this invention applies include, for example, maize, wheat, rice, barley, oats, rye, and millet.

The subject invention also concerns methods for producing and identifying polynucleotides and polypeptides contemplated within the scope of the invention. In one embodiment, gene mutation, followed by selection using a bacterial expression system, can be used to isolate polynucleotide molecules that encode plant AGP subunits that possess mutations that can alleviate heat-induced loss in starch synthesis in plants. Individual amino acid substitutions can be combined into one subunit as described herein.

The subject invention further concerns plants and plant tissue that comprise a polynucleotide of the present invention that encodes a mutant polypeptide of the invention. In a preferred embodiment, the plant or plant tissue has an AGP mutant gene of the invention incorporated into its genome. Other alleles that confer advantageous phenotypes can also be incorporated into a plant genome. In a preferred embodiment, the plant is a cereal plant. More preferably, the plant is *Zea mays*. Plants having an AGP mutant gene can be grown from seeds that comprise a mutant gene in their genome. In addition, techniques for transforming plants with a gene, such as *Agrobacterium* infection, biolistic methods, *etc.*, are known in the art.

Because of the degeneracy of the genetic code, a variety of different polynucleotide sequences can encode each of the variant AGP polypeptides disclosed herein. In addition, it is well within the skill of a person trained in the art to create alternative polynucleotide sequences encoding the same, or essentially the same, polypeptides of the subject invention. These variant or alternative polynucleotide sequences are within the scope of the subject invention. As used herein, references to "essentially the same" sequence refers to sequences which encode amino acid substitutions, deletions, additions, or insertions which do not materially alter the functional activity of the polypeptide encoded by the AGP mutant polynucleotide described herein.

As used herein, the terms "nucleic acid" and "polynucleotide sequence" refer to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally-occurring nucleotides. The polynucleotide sequences include both the DNA strand sequence that is transcribed into RNA and the RNA sequence that is translated into protein. The polynucleotide sequences include both

full-length sequences as well as shorter sequences derived from the full-length sequences. It is understood that a particular polynucleotide sequence includes the degenerate codons of the native sequence or sequences which may be introduced to provide codon preference in a specific host cell. Allelic variations of the exemplified sequences also come within the scope of the subject invention. The polynucleotide sequences falling within the scope of the subject invention further include sequences which specifically hybridize with the exemplified sequences. The polynucleotide includes both the sense and antisense strands as either individual strands or in the duplex.

Substitution of amino acids other than those specifically exemplified in the mutants disclosed herein are also contemplated within the scope of the present invention. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby a mutant AGP polypeptide having an amino acid of one class is replaced with another amino acid of the same class fall within the scope of the subject invention so long as the mutant AGP polypeptide having the substitution still retains increased heat stability relative to a wild type polypeptide. Table 2 below provides a listing of examples of amino acids belonging to each class.

Table 2.

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

For example, substitution of the tyrosine at position 333 in the HS 33, HS 7+3, HS 6+3, and HS 7+6+3 mutants with other amino acids, such as glycine, serine, threonine, cysteine, asparagine, and glutamine, are encompassed within the scope of the invention. Also specifically contemplated within the scope of the invention is substitution of either a phenylalanine or a methionine at position 333 in the AGP large subunit. Thus, a combination of phenylalanine or methionine at position 333 with either a valine at position 177 or a valine at position 396, or both, is specifically contemplated by the present

invention. Similarly, substitution of the valine at positions 177 and 396 in the RTS 48-2, RTS 60-1, HS 7+3, HS 6+3, HS 7+6, and HS 7+6+3 mutants with other amino acids such as leucine, isoleucine, proline, methionine, phenylalanine, and tryptophan, is within the scope of the invention. Amino acid substitutions at positions other than the site of the heat stable mutation are also contemplated within the scope of the invention so long as the polypeptide retains or confers increased heat stability relative to wild type polypeptides.

Polynucleotides and proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges with those exemplified herein. The sequence identity will typically be greater than 60%, preferably greater than 75%, more preferably greater than 80%, even more preferably greater than 90%, and can be greater than 95%. The identity and/or similarity of a sequence can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified herein. Unless otherwise specified, as used herein percent sequence identity and/or similarity of two sequences can be determined using the algorithm of Karlin and Altschul (Karlin and Altschul [1990] *Proc. Natl. Acad. Sci. USA* 87:2264-2268), modified as in Karlin and Altschul (Karlin and Altschul [1993] *Proc. Natl. Acad. Sci. USA* 90:5873-5877). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.* (Altschul *et al.* [1990] *J. Mol. Biol.* 215:402-410). BLAST searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain sequences with the desired percent sequence identity. To obtain gapped alignments for comparison purposes, Gapped BLAST can be used as described in Altschul *et al.* (Altschul *et al.* [1997] *Nucl. Acids Res.* 25:3389-3402). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) can be used. *See* NCBI/NIH website.

The subject invention also concerns polynucleotides which encode fragments of the full length mutant polypeptide, so long as those fragments retain substantially the same functional activity as full length polypeptide. The fragments of mutant AGP polypeptide encoded by these polynucleotides are also within the scope of the present invention.

Fragments of the full length sequence can be prepared using standard techniques known in the art.

The subject invention also contemplates those polynucleotide molecules encoding starch biosynthesis enzymes having sequences which are sufficiently homologous with the wild type sequence so as to permit hybridization with that sequence under standard stringent conditions and standard methods (Maniatis, T., E.F. Fritsch, J. Sambrook [1982] *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). As used herein, "stringent" conditions for hybridization refers to conditions wherein hybridization is typically carried out overnight at 20-25°C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz, G.A., K.A. Jacobs, T.H. Eickbush, P.T. Cherbas, and F.C. Kafatos [1983] *Methods of Enzymology*, R. Wu, L. Grossman and K. Moldave [eds.] Academic Press, New York 100:266-285):

Tm=81.5°C+16.6 Log[Na+]+0.41(%G+C)-0.61(% formamide)-600/length of duplex in base pairs.

Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20°C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

The polynucleotide molecules of the subject invention can be used to transform plants to express the mutant heat stable enzyme in those plants. In addition, the polynucleotides of the subject invention can be used to express the recombinant variant enzyme. They can also be used as a probe to detect related enzymes. The polynucleotides can also be used as DNA sizing standards.

The polynucleotide molecules of the subject invention also include those polynucleotides that encode starch biosynthesis enzymes, such as AGP enzymes, that contain mutations that can confer increased seed weight, in addition to enhanced heat stability, to a plant expressing these mutants. The combination of a heat stabilizing mutation, such as, for example, *Sh2-HS 7+6* or *Sh2-HS 7+3*, with a mutation conferring

increased seed weight, *e.g.*, *Rev6*, in a polynucleotide that encodes the large subunit of maize AGP is specifically contemplated in the present invention. U.S. Patent Nos. 5,589,618 and 5,650,557 disclose polynucleotides (*e.g.*, *Rev6*) that encode mutations in the large subunit of AGP that confer increased seed weight in plants that express the mutant polypeptide.

Mutations in the AGP subunits that confer heat stability can be combined according to the subject invention with phosphate insensitive mutants of maize, such as the *Rev6* mutation, to enhance the stability of the *Rev6* encoded large subunit.

It is expected that enzymic activity of SSS will be impaired at higher temperatures as observed with AGP. Thus, mutagenized forms of SSS can be expressed under increased thermal conditions (42°C), to isolate heat stable variants in accordance with the methods described herein. These heat stable mutagenized forms of SSS are further aspects of the subject invention.

The subject invention also concerns methods for increasing yield characteristics of plants under conditions of heat stress by incorporating a polynucleotide of the present invention that comprises a mutation in a starch biosynthesis enzyme that confers increased stability or resistance to heat stress conditions and a mutation that confers increased yield characteristics on the plant. Increased yield characteristics include, for example, increased seed number, increased seed weight, increased plant biomass, and increased Harvest Index.

All patents, patent applications, provisional applications, and publications referred to or cited herein are hereby incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 - Testing of Maize Endosperm ADP-glucose Pyrophosphorylases having Multiple Amino Acid Mutations

Expression of Maize Endosperm ADP-glucose Pyrophosphorylases. 10-ml aliquots of Luria broth (75 g/mL of spectinomycin and 50 g/mL of kanamycin) were inoculated from glycerol stocks of AC70R1-504 *E. coli* cells expressing either maize endosperm or potato tuber ADP-glucose pyrophosphorylase, and grown overnight at 37C with shaking at 220 rpm. These cultures were used to inoculate 250 mL of Luria broth (75 g/mL of spectinomycin and 50 g/mL of kanamycin). Cultures were grown to an $OD_{600} = 0.55$ at 37C with shaking at 220 rpm. Cultures were induced with 0.2 mM isopropyl -D-thiogalactoside and .02 mg/mL nalidixic acid for 7 hrs at room temperature with shaking at 220 rpm. Cells were harvested at 3500 rpm for 10 min at 4C. Cell pellets were resuspended in 800L of extraction buffer: 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 20% sucrose, and 30% ammonium sulfate. DTT (1 mM), 50 g/ml lysozyme, 1 g/mL pepstatin, 1 g/mL leupeptin, 1 g/mL antipain, 10 g/mL chymostatin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine were added to extraction buffer just before use. Lysates were sonicated three times for three seconds with incubation on ice between sonications. Samples were centrifuged for 1 min at 13,000 rpm at 4C. Supernatants were removed and aliquoted for assays.

Combination of Individual Mutations. A subcloning strategy was designed to study the effects of the mutations in combination with *HS 33*, and with each other. To combine the reversion mutations of *RTS 48-2* and *RTS 60-1*, the plasmids containing each reversion mutation (the temperature sensitive parental mutations were removed prior to combining mutations) were digested with *Eco* RV and a 339 bp fragment of *RTS 48-2* was exchanged for the corresponding fragment of *RTS 60-1* (Figure 3). The resulting plasmid was designated *Sh2-HS 7+6*. A similar strategy was used to combine the reversion mutation of *RTS 48-2* with the mutation identified in *HS 33*. Plasmids containing the mutations were digested with *Eco* RV and a 339 bp fragment of *RTS 48-2* was exchanged for the corresponding fragment of *HS 33*. The resulting plasmid was designated *Sh2-HS 7+3*. To combine the reversion mutation of *RTS 60-1* with the mutation identified in *HS 33*, plasmids containing the mutations were digested with *Mun* I/*Kpn* I and a 390 bp fragment of *RTS 60-1* was exchanged for the corresponding fragment of *HS 33* (Figure

3). The resulting plasmid was designated *Sh2-HS 6+3*. In order to combine the reversion mutations of *RTS 60-1* and *RTS 48-2* with the mutation identified in *HS 33*, plasmid *Sh2-HS 6+3* and a plasmid containing the reversion mutation *RTS 48-2* were digested with *Eco* RV and a 339 bp fragment of *RTS 48-2* was exchanged for the corresponding fragment of *Sh2-HS 6+3*. The resulting plasmid was designated *Sh2-HS 7+6+3*.

Final sequencing of all plasmids was performed using six primers to cover the entire *Sh2* coding region in both directions. Primers used are as follows:

LHBB1 (5'→3'): 5'-CGACTCACTATAGGGAGACC-3' (SEQ ID NO. 5);

LH27 (5'→3'): 5'-CCCTATGAGTAACTG-3' (SEQ ID NO. 6);

LH9 (5'→3'): 5'-TATACTCAATTACAT-3' (SEQ ID NO. 7);

LHBB2 (3'→5'): 5'-GTGCCACCTGACGTCTAAG-3' (SEQ ID NO. 8);

LH2135 (3'→5'): 5'-CAGAGCTGACACGTG-3' (SEQ ID NO. 9);

LH32 (3'→5'): 5'-AAGCTGATCGCCACTC-3' (SEQ ID NO. 10).

Heat Treatment of ADP-glucose Pyrophosphorylase. Wild type (sh2) and mutant ADP-glucose pyrophosphorylase containing a single amino acid mutation (HS 33, RTS 48-2, RTS 60-1) and multiple mutation (HS 7+3, *i.e.*, RTS 48-2 plus HS 33; HS 6+3, *i.e.*, RTS 60-1 plus HS 33; and HS 7+6, *i.e.*, RTS 48-2 plus RTS 60-1) amino acid changes were tested for enzyme activity before and after heat treatment. Heat treatment consisted of incubation of the test protein at 60°C for 5 minutes.

The percentage of activity remaining after heat treatment at 60°C for 5 min is presented in Table 3. Genotypes in the data set are *Sh2* wild type, *HS 33*, *RTS 60-1* (reversion mutation only), *RTS 48-2* (reversion mutation only), *Sh2-HS 7+6*, *Sh2-HS 6+3*, *Sh2-HS 7+3*, and *Sh2-HS 7+6+3*.

Table 3. Percent Activity Remaining After Heat Treatment

Enzyme	% Activity	SEM[a]	N[b]
Sh2 wt	32	11	3
HS 33	69	7	7
RTS 60-1	61	13*	2
RTS 48-2	64	6	3
Sh2-HS 7+6	77	21*	2
Sh2-HS 6+3	69	9	3
Sh2-HS 7+3	83	8	3
Sh2-HS 7+6+3	72	11	3

[a] standard error of the mean
[b] number of experimental replicates
* represents range, rather than S.E.M.

Activity before heat treatment for *Sh2* wild type, *HS 33*, *RTS 60-1*, *RTS 48-2*, *Sh2-HS 7+6*, *Sh2-HS 6+3*, *Sh2-HS 7+3*, and *Sh2-HS 7+6+3* is shown in Table 4. *HS 33* has 2.1 fold more activity than does *Sh2* wild type. Both *RTS 48-2* and *RTS 60-1* show a 1.4 fold increase in activity. Their double mutant contains a 1.9 fold increase in activity. While the combination of the two mutants increases activity, the double mutant does not experience synergistic effects. The mutation of *RTS 60-1* when combined with that of *HS 33* experiences an additive effect, raising activity 3.4 fold compared to *Sh2* wild type. The mutation of *RTS 48-2* in combination with that of *HS 33* exhibits a slightly smaller increase to 2.9 fold. Interestingly, the triple mutant shows a slightly greater increase than either second-site reversion mutation alone, but less than the double mutant between second-site revertants.

Table 4. Fold Increase in Activity

Enzyme	Fold increase	Range	N[b]
Sh2 wt	n/a	n/a	n/a
HS 33	2.1	0.2[a]	3
RTS 60-1	1.4	0	1
RTS 48-2	1.4	0	1
Sh2-HS 7+6	1.9	0.2	2
Sh2-HS 6+3	3.4	0	1
Sh2-HS 7+3	2.9	0.1	2
Sh2-HS 7+6+3	1.8	0.1	2

[a] standard error of the mean
[b] number of experimental replicates
n/a not applicable

ADP-glucose Pyrophosphorylase Assays. To obtain quantitative data for the mutants described above, activity was measured with the synthesis (forward) assay that measures incorporation of [^{14}C]glucose-1-P into the sugar nucleotide ADP-glucose. Assays were performed on crude enzyme extracts prepared as described below.

The ADP-glucose synthesis reaction measures incorporation of [^{14}C]glucose-1-P into ADP-glucose. The reaction mixture contained 80 mM HEPES, pH 7.5m, 1 mM glucose-1-P, 4 mM $MgCl_2$, 0.5 mg mL^{-1} bovine serum albumin, 10 mM 3-PGA, and 15,000 cpm of [^{14}C]glucose-1-P. Reaction volume was 50 mL. Assays were initiated by addition of 1.5 mM ATP. Reaction was incubated for 30 min at 37°C and terminated by boiling for 2 min. Unincorporated glucose-1-P was cleaved by addition of 0.3 U of bacterial alkaline phosphatase (Worthington Biochemical Corporation, Lakewood, NJ) and incubation for 2.5 h at 37°C. A 20 mL aliquot of the reaction mixture was spotted on DEAE paper, washed with distilled water three times, dried, and quantified in a liquid scintillation counter.

Additional results for single and double mutants are shown in Figure 2. For the combination mutant *Sh2-HS 7+6* (*RTS 48-2* plus *RTS 60-1*) and the combination mutant

Sh2-HS 6+3 (*RTS 60-1* plus *HS 33*), the numbers given are the average of data from 3 dilutions of the enzyme (in duplicate), multiplied by their dilution factor, minus background. For the combination *Sh2-HS 7+3* (*RTS 48-2* plus *HS 33*) mutant, the numbers given are the average of 2 dilutions of the enzyme (in duplicate), multiplied by their dilution factor, minus background. Graphic representation of the numbers was performed using Microsoft Excel.

Example 2 – Combination of Heat Stability Mutations with *Rev6*

According to the subject invention, the heat stable mutations can be combined with a mutation associated with increased seed weight, such as, for example, the *Rev6* mutation. The goal is to maintain the desired phosphate insensitivity characteristic of *Rev6* while enhancing its stability. Mutants comprising heat stable mutations combined with *Rev6* mutation can be constructed and confirmed as described herein. These "combination" mutants can be transformed into AC70R1-504 carrying the wild type small subunit. Increased heat stability can be easily identified by a positive glycogen staining on a low glucose media. *Rev6* does not stain when grown on this media. Initially all mutant combinations can be screened enzymatically for maintenance of phosphate insensitivity, and only combinations that maintain phosphate insensitivity are further analyzed.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

Claims

We claim:

1. A polynucleotide encoding a mutant large subunit of a plant ADP-glucose pyrophosphorylase polypeptide, or a biologically-active fragment of said mutant polypeptide, wherein said mutant polypeptide comprises amino acid mutations at two or more sites in the amino acid sequence of said polypeptide and wherein when said mutant polypeptide is expressed with the small subunit of ADP-glucose pyrophosphorylase to form a mutant ADP-glucose pyrophosphorylase enzyme, said mutant enzyme, or a biologically-active fragment of said mutant enzyme, exhibits increased heat stability relative to wild type ADP-glucose pyrophosphorylase enzyme.

2. The polynucleotide according to claim 1, wherein said mutant enzyme exhibits enzymatic activity substantially the same or greater than that exhibited by an ADP-glucose pyrophosphorylase enzyme having only a single amino acid substitution of a histidine to tyrosine at position 333 in the amino acid sequence of the wild type large subunit of maize.

3. The polynucleotide according to claim 1, wherein said mutant polypeptide encoded by said polynucleotide comprises a first amino acid mutation wherein the histidine amino acid corresponding to position 333 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

4. The polynucleotide according to claim 3, wherein the amino acid that replaces histidine at position number 333 is selected from the group consisting of tyrosine, phenylalanine, methionine, glycine, serine, threonine, cysteine, asparagine, and glutamine.

5. The polynucleotide according to claim 3, wherein the amino acid that replaces histidine at position number 333 is tyrosine.

6. The polynucleotide according to claim 3, wherein the amino acid that replaces histidine at position number 333 is phenylalanine.

7. The polynucleotide according to claim 3, wherein the amino acid that replaces histidine at position number 333 is methionine.

8. The polynucleotide according to claim 1, wherein said mutant polypeptide encoded by said polynucleotide comprises a first amino acid mutation wherein the alanine amino acid corresponding to position 177 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

9. The polynucleotide according to claim 8, wherein the amino acid that replaces alanine at position number 177 is a proline.

10. The polynucleotide according to claim 8, wherein the amino acid that replaces alanine at position number 177 is a valine.

11. The polynucleotide according to claim 1, wherein said mutant polypeptide encoded by said polynucleotide comprises a first amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme

12. The polynucleotide according to claim 11, wherein the amino acid that replaces alanine at position number 396 is a valine.

13. The polynucleotide according to claim 3, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 177 in the amino acid sequence of the wild

type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

14. The polynucleotide according to claim 13, wherein the amino acid that replaces alanine at position number 177 is a proline.

15. The polynucleotide according to claim 13, wherein the amino acid that replaces alanine at position number 177 is a valine.

16. The polynucleotide according to claim 4, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 177 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

17. The polynucleotide according to claim 16, wherein the amino acid that replaces alanine at position number 177 is a proline.

18. The polynucleotide according to claim 16, wherein the amino acid that replaces alanine at position number 177 is a valine.

19. The polynucleotide according to claim 5, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 177 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

20. The polynucleotide according to claim 19, wherein the amino acid that replaces alanine at position number 177 is a proline.

21. The polynucleotide according to claim 19, wherein the amino acid that replaces alanine at position number 177 is a valine.

22. The polynucleotide according to claim 6, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 177 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

23. The polynucleotide according to claim 22, wherein the amino acid that replaces alanine at position number 177 is a proline.

24. The polynucleotide according to claim 22, wherein the amino acid that replaces alanine at position number 177 is a valine.

25. The polynucleotide according to claim 7, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 177 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

26. The polynucleotide according to claim 25, wherein the amino acid that replaces alanine at position number 177 is a proline.

27. The polynucleotide according to claim 25, wherein the amino acid that replaces alanine at position number 177 is a valine.

28. The polynucleotide according to claim 3, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild

21. The polynucleotide according to claim 19, wherein the amino acid that replaces alanine at position number 177 is a valine.

22. The polynucleotide according to claim 6, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 177 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

23. The polynucleotide according to claim 22, wherein the amino acid that replaces alanine at position number 177 is a proline.

24. The polynucleotide according to claim 22, wherein the amino acid that replaces alanine at position number 177 is a valine.

25. The polynucleotide according to claim 7, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 177 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

26. The polynucleotide according to claim 25, wherein the amino acid that replaces alanine at position number 177 is a proline.

27. The polynucleotide according to claim 25, wherein the amino acid that replaces alanine at position number 177 is a valine.

28. The polynucleotide according to claim 3, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild

type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

29. The polynucleotide according to claim 28, wherein the amino acid that replaces alanine at position number 396 is a valine.

30. The polynucleotide according to claim 4, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

31. The polynucleotide according to claim 30, wherein the amino acid that replaces alanine at position number 396 is a valine.

32. The polynucleotide according to claim 5, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

33. The polynucleotide according to claim 32, wherein the amino acid that replaces alanine at position number 396 is a valine.

34. The polynucleotide according to claim 6, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

35. The polynucleotide according to claim 34, wherein the amino acid that replaces alanine at position number 396 is a valine.

36. The polynucleotide according to claim 7, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

37. The polynucleotide according to claim 36, wherein the amino acid that replaces alanine at position number 396 is a valine.

38. The polynucleotide according to claim 8, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

39. The polynucleotide according to claim 38, wherein the amino acid that replaces alanine at position number 396 is a valine.

40. The polynucleotide according to claim 9, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

41. The polynucleotide according to claim 40, wherein the amino acid that replaces alanine at position number 396 is a valine.

42. The polynucleotide according to claim 10, wherein said mutant polypeptide encoded by said polynucleotide comprises a second amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

43. The polynucleotide according to claim 42, wherein the amino acid that replaces alanine at position number 396 is a valine.

44. The polynucleotide according to claim 13, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

45. The polynucleotide according to claim 44, wherein the amino acid that replaces alanine at position 396 is a valine.

46. The polynucleotide according to claim 14, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

47. The polynucleotide according to claim 46, wherein the amino acid that replaces alanine at position 396 is a valine.

48. The polynucleotide according to claim 15, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type

large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

49. The polynucleotide according to claim 48, wherein the amino acid that replaces alanine at position 396 is a valine.

50. The polynucleotide according to claim 16, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

51. The polynucleotide according to claim 50, wherein the amino acid that replaces alanine at position 396 is a valine.

52. The polynucleotide according to claim 17, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

53. The polynucleotide according to claim 52, wherein the amino acid that replaces alanine at position 396 is a valine.

54. The polynucleotide according to claim 18, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

55. The polynucleotide according to claim 54, wherein the amino acid that replaces alanine at position 396 is a valine.

56. The polynucleotide according to claim 19, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

57. The polynucleotide according to claim 56, wherein the amino acid that replaces alanine at position 396 is a valine.

58. The polynucleotide according to claim 20, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

59. The polynucleotide according to claim 58, wherein the amino acid that replaces alanine at position 396 is a valine.

60. The polynucleotide according to claim 21, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

61. The polynucleotide according to claim 60, wherein the amino acid that replaces alanine at position 396 is a valine.

62. The polynucleotide according to claim 22, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

63. The polynucleotide according to claim 62, wherein the amino acid that replaces alanine at position 396 is a valine.

64. The polynucleotide according to claim 23, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

65. The polynucleotide according to claim 64, wherein the amino acid that replaces alanine at position 396 is a valine.

66. The polynucleotide according to claim 24, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

67. The polynucleotide according to claim 66, wherein the amino acid that replaces alanine at position 396 is a valine.

68. The polynucleotide according to claim 25, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type

large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

69. The polynucleotide according to claim 68, wherein the amino acid that replaces alanine at position 396 is a valine.

70. The polynucleotide according to claim 26, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

71. The polynucleotide according to claim 70, wherein the amino acid that replaces alanine at position 396 is a valine.

72. The polynucleotide according to claim 27, wherein said mutant polypeptide encoded by said polynucleotide comprises a third amino acid mutation wherein the alanine amino acid corresponding to position 396 in the amino acid sequence of the wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize is replaced by an amino acid that confers said increased heat stability on said mutant enzyme.

73. The polynucleotide according to claim 72, wherein the amino acid that replaces alanine at position 396 is a valine.

74. The polynucleotide according to claim 1, wherein said mutant protein encoded by said polynucleotide further comprises an amino acid mutation that confers increased seed weight to a plant expressing said polynucleotide.

75. The polynucleotide according to claim 74, wherein said polynucleotide comprises the *Rev6* mutation.

76. The polynucleotide according to claim 74, wherein said polynucleotide encodes a large subunit AGP enzyme wherein at least one serine residue is inserted between the amino acids corresponding to 494 and 495 in the amino acid sequence of wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize of the native AGP enzyme subunit.

77. The polynucleotide according to claim 74, wherein said polynucleotide encodes a large subunit AGP enzyme wherein the amino acid pair tyrosine:serine is inserted between the amino acids corresponding to 494 and 495 in the amino acid sequence of wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize of the native AGP enzyme subunit.

78. The polynucleotide according to claim 74, wherein said polynucleotide encodes a large subunit AGP enzyme wherein the amino acid pair serine:tyrosine is inserted between the amino acids corresponding to 495 and 496 in the amino acid sequence of wild type large subunit of ADP-glucose pyrophosphorylase polypeptide of maize of the native AGP enzyme subunit.

79. A method for increasing resistance of a plant to heat stress conditions, said method comprising incorporating a polynucleotide selected from the group consisting of the polynucleotide of claim 1, the polynucleotide of claim 2, the polynucleotide of claim 3, the polynucleotide of claim 4, the polynucleotide of claim 5, the polynucleotide of claim 6, the polynucleotide of claim 7, the polynucleotide of claim 8, the polynucleotide of claim 9, the polynucleotide of claim 10, the polynucleotide of claim 11, the polynucleotide of claim 12, the polynucleotide of claim 13, the polynucleotide of claim 14, the polynucleotide of claim 15, the polynucleotide of claim 16, the polynucleotide of claim 17, the polynucleotide of claim 18, the polynucleotide of claim 19, the polynucleotide of claim 20, the polynucleotide of claim 21, the polynucleotide of claim 22, the polynucleotide of claim 23, the polynucleotide of claim 24, the polynucleotide of claim 25, the polynucleotide of claim 26, the polynucleotide of claim 27, the polynucleotide of claim 28, the polynucleotide of claim 29, the polynucleotide of claim 30, the polynucleotide of claim

31, the polynucleotide of claim 32, the polynucleotide of claim 33, the polynucleotide of claim 34, the polynucleotide of claim 35, the polynucleotide of claim 36, the polynucleotide of claim 37, the polynucleotide of claim 38, the polynucleotide of claim 39, the polynucleotide of claim 40, the polynucleotide of claim 41, the polynucleotide of claim 42, the polynucleotide of claim 43, the polynucleotide of claim 44, the polynucleotide of claim 45, the polynucleotide of claim 46, the polynucleotide of claim 47, the polynucleotide of claim 48, the polynucleotide of claim 49, the polynucleotide of claim 50, the polynucleotide of claim 51, the polynucleotide of claim 52, the polynucleotide of claim 53, the polynucleotide of claim 54, the polynucleotide of claim 55, the polynucleotide of claim 56, the polynucleotide of claim 57, the polynucleotide of claim 58, the polynucleotide of claim 59, the polynucleotide of claim 60, the polynucleotide of claim 61, the polynucleotide of claim 62, the polynucleotide of claim 63, the polynucleotide of claim 64, the polynucleotide of claim 65, the polynucleotide of claim 66, the polynucleotide of claim 67, the polynucleotide of claim 68, the polynucleotide of claim 69, the polynucleotide of claim 70, the polynucleotide of claim 71, the polynucleotide of claim 72, and the polynucleotide of claim 73 in said plant and expressing the protein encoded by said polynucleotide.

80. The method according to claim 79, wherein said plant is a monocotyledonous plant.

81. The method according to claim 80, wherein said monocotyledonous plant is selected from the group consisting of rice, wheat, barley, oats, sorghum, maize, lilies, and millet.

82. The method according to claim 79, wherein said plant is *Zea mays*.

83. The method according to claim 79, wherein said plant is a dicotyledonous plant.

84. The method according to claim 83, wherein said dicotyledonous plant is selected from the group consisting of peas, alfalfa, chickpea, chicory, clover, kale, lentil, prairie grass, soybean, tobacco, potato, sweet potato, radish, cabbage, rape, apple trees, and lettuce.

85. A plant or plant tissue comprising a polynucleotide selected from the group consisting of the polynucleotide of claim 1, the polynucleotide of claim 2, the polynucleotide of claim 3, the polynucleotide of claim 4, the polynucleotide of claim 5, the polynucleotide of claim 6, the polynucleotide of claim 7, the polynucleotide of claim 8, the polynucleotide of claim 9, the polynucleotide of claim 10, the polynucleotide of claim 11, the polynucleotide of claim 12, the polynucleotide of claim 13, the polynucleotide of claim 14, the polynucleotide of claim 15, the polynucleotide of claim 16, the polynucleotide of claim 17, the polynucleotide of claim 18, the polynucleotide of claim 19, the polynucleotide of claim 20, the polynucleotide of claim 21, the polynucleotide of claim 22, the polynucleotide of claim 23, the polynucleotide of claim 24, the polynucleotide of claim 25, the polynucleotide of claim 26, the polynucleotide of claim 27, the polynucleotide of claim 28, the polynucleotide of claim 29, the polynucleotide of claim 30, the polynucleotide of claim 31, the polynucleotide of claim 32, the polynucleotide of claim 33, the polynucleotide of claim 34, the polynucleotide of claim 35, the polynucleotide of claim 36, the polynucleotide of claim 37, the polynucleotide of claim 38, the polynucleotide of claim 39, the polynucleotide of claim 40, the polynucleotide of claim 41, the polynucleotide of claim 42, the polynucleotide of claim 43, the polynucleotide of claim 44, the polynucleotide of claim 45, the polynucleotide of claim 46, the polynucleotide of claim 47, the polynucleotide of claim 48, the polynucleotide of claim 49, the polynucleotide of claim 50, the polynucleotide of claim 51, the polynucleotide of claim 52, the polynucleotide of claim 53, the polynucleotide of claim 54, the polynucleotide of claim 55, the polynucleotide of claim 56, the polynucleotide of claim 57, the polynucleotide of claim 58, the polynucleotide of claim 59, the polynucleotide of claim 60, the polynucleotide of claim 61, the polynucleotide of claim 62, the polynucleotide of claim 63, the polynucleotide of claim 64, the polynucleotide of claim 65, the polynucleotide of claim 66, the polynucleotide of claim 67, the polynucleotide of claim 68, the polynucleotide of claim

69, the polynucleotide of claim 70, the polynucleotide of claim 71, the polynucleotide of claim 72, and the polynucleotide of claim 73.

86. The plant or plant tissue according to claim 85, wherein said plant or plant tissue is monocotyledonous.

87. The plant or plant tissue according to claim 86, wherein said monocotyledonous plant or plant tissue is selected from the group consisting of rice, wheat, barley, oats, sorghum, maize, lilies, and millet.

88. The plant or plant tissue according to claim 85, wherein said plant is *Zea mays* or said plant tissue is from *Zea mays*.

89. The plant or plant tissue according to claim 85, wherein said plant or plant tissue is dicotyledonous.

90. The plant or plant tissue according to claim 89, wherein said dicotyledonous plant or plant tissue is selected from the group consisting of peas, alfalfa, chickpea, chicory, clover, kale, lentil, prairie grass, soybean, tobacco, potato, sweet potato, radish, cabbage, rape, apple trees, and lettuce.

91. The plant tissue according to claim 85, wherein said plant tissue is a seed.

92. A mutant starch biosynthesis protein encoded by the polynucleotide of claim 1.

93. A method for increasing a characteristic of a plant selected from the group consisting of seed number, plant biomass, Harvest Index, flag leaf weight, seed heads, and total seed weight, said method comprising incorporating the polynucleotide of claim 75 into the genome of said plant and expressing the protein encoded by said polynucleotide molecule.

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-1020 TGATGCTTTTTCCTGGGCAGGGAGAGCTATGAGACGTATGTCCTCAAAGCCACTTTGCAT
 -960 TGTGTGAAACCAATATCGATCTTTGTTACTTCATCATGCATGAACATTTGTGGAAACTAC
 -900 TAGCTTACAAGCATTAGTGACAGCTCAGAAAAAAGTTATCTCTGAAAGGTTTCATGTGTA
 -840 CCGTGGGAAATGAGAAATGTTGCCAACTCAAACACCTTCAATATGTTGTTTGCAGGCAAA
 -780 CTCTTCTGGAAGAAAGGTGTCTAAAACTATGAACGGGTTACAGAAAGGTATAAACCACGG
 -720 CTGTGCATTTTGGAAGTATCATCTATAGATGTCTGTTGAGGGGAAAGCCGTACGCCAACG
 -660 TTATTTACTCAGAAACAGCTTCAACACACAGTTGTCTGCTTTATGATGGCATCTCCACCC
 -600 AGGCACCCACCATCACCTATTCACCTATCTCTCGTGCCTGTTTATTTTCTTGCCCTTTCT
 -540 GATCATAAAAAATCATTAAGAGTTTGCAAACATGCATAGGCATATCAATATGCTCATTTA
 -480 TTAATTTGCTAGCAGATCATCTTCCTACTCTTTACTTTATTTATTGTTTGAAAAATATGT
 -420 CCTGCACCTAGGGAGCTCGTATACAGTACCAATGCATCTTCATTAAATGTGAATTTCAGA
 -360 AAGGAAGTAGGAACCTATGAGAGTATTTTTCAAAATTAATTAGCGGCTTCTATTATGTTT
 -300 ATAGCAAAGGCCAAGGGCAAAATCGGAACACTAATGATGGTTGGTTGCATGAGTCTGTCG
 -240 ATTACTTGCAAGAAATGTGAACCTTTGTTTCTGTGCGTGGGCATAAAACAAACAGCTTCT
 -180 AGCCTCTTTTACGGTACTTGCACTTGCAAGAAATGTGAACTCCTTTTCATTTCTGTATGT
 -120 GGACATAATGCCAAAGCATCCAGGCTTTTTCATGGTTGTTGATGTCTTTACACAGTTCAT
  -60 CTCCACCAGTATGCCCTCCTCATACTCTATATAAACACATCAACAGCATCGCAATTAGCC
    1 ACAAGATCACTTCGGGAGGCAAGTGTGATTTCGACCTTGCAGCCACCTTTTTTTGTTCTG
   61 TTgtaagtatactttcccttaccatctttatctgttagtttaatttgtaattgggaagta
  121 ttagtggaaagaggatgagatgctatcatctatgtactctgcaaatgcatctgacgttat
  181 atgggctgcttcatataatttgaattgctccattcttgccgacaatatattgcaaggtat
  241 atgcctagttccatcaaaagttctgtttttttcattctaaaagcattttagtggcacgcaa
  301 ttttgtccatgagggaaaggaaatctgttttggttactttgcttgaggtgcattcttcat
  361 atgtccagttttatggaagtaataaacttcagtttggtcataagatgtcatattaaaggg
  421 caaacatatattcaatgttcaattcatcgtaaatgttccctttttgtaaaagattgcata
  481 ctcatttatttgagttgcagGTGTATCTAGTAGTTGGAGGAGATATGCAGTTTGCACTTG
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FIG. 1A

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                                                    M  Q  F  A  L
541 CATTGGACACGAACTCAGGTCCTCACCAGATAAGATCTTGTGAGGGTGATGGGATTGACA
    A  L  D  T  N  S  G  P  H  Q  I  R  S  C  E  G  D  G  I  D
601 GGTTGGAAAAATTAAGTATTGGGGGCAGAAAGCAGGAGAAAGCTTTGAGAAATAGGTGCT
    R  L  E  K  L  S  I  G  G  R  K  Q  E  K  A  L  R  N  R  C
661 TTGGTGGTAGAGTTGCTGCAACTACACAATGTATTCTTACCTCAGATGCTTGTCCTGAAA
    F  G  G  R  V  A  A  T  T  Q  C  I  L  T  S  D  A  C  P  E
721 CTCTTgtaagtatccacctcaattattactcttacatgttggtttactttacgtttgtct
    T  L
781 tttcaagggaaatttactgtatttttgtgttttgtgggagttctatacttctgttggac
841 tggttattgtaaagatttgttcaaatagggtcatctaataattgtttgaaatctgggaac
901 tgtggtttcactgcgttcaggaaaaagtgaattattggttactgcatgaataacttatgg
961 aaatagaccttagagttgctgcatgattatcacaaatcattgctacgatatcttataata
1021 gttctttcgacctcgcattacatatataactgcaactcctagttgcgttcaaaaaaaaaa
1081 atgcaactcttagaacgctcaccagtgtaatctttcctgaattgttatttaatggcatgt
1141 atgcactacttgtatacttatctaggattaagtaatctaactctaggccccatatttgca
1201 gCATTCTCAAACACAGTCCTCTAGGAAAAATTATGCTGATGCAAACCGTGTATCTGCTAT
      H  S  Q  T  Q  S  S  R  K  N  Y  A  D  A  N  R  V  S  A  I
1261 CATTTTGGGCGGAGGCACTGGATCTCAGCTCTTTCCTCTGACAAGCACAAGAGCTACGCC
      I  L  G  G  G  T  G  S  Q  L  F  P  L  T  S  T  R  A  T  P
1321 TGCTgtaagggataacactgaacatccaacgttgattactctattatagtattatacaga
      A
1381 ctgtacttttcgaatttatcttagttttctacaatatttagtggattcttctcattttca
1441 agatacacaattgatccataatcgaagtggtatgtaagacagtgagttaaaagattatat
1501 tttttgggagacttccagtcaaattttcttagaagtttttttggtccagatgttcataaa
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FIG. 1B

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1561 gtcgccgctttcatacttttttaatttttaattggtgcactattagGTACCTGTTGGA
                                                    V  P  V  G
1621 GGATGTTACAGGCTTATTGATATCCCTATGAGTAACTGCTTCAACAGTGGTATAAATAAG
      G  C  Y  R  L  I  D  I  P  M  S  N  C  F  N  S  G  I  N  K
1681 ATATTTGTGATGAGTCAGTTCAATTCTACTTCGCTTAACCGCCATATTCATCGTACATAC
      I  F  V  M  S  Q  F  N  S  T  S  L  N  R  H  I  H  R  T  Y
1741 CTTGAAGGCGGGATCAACTTTGCTGATGGATCTGTACAGgtgatttacctcatcttgttg
      L  E  G  G  I  N  F  A  D  G  S  V  Q
1801 atgtgtaatactgtaattaggagtagatttgtgtggagagaataataaacagatgccgag
1861 attcttttctaaaagtctagatccaaaggcattgtggttcaaaacactatggacttctac
1921 catttatgtcattactttgccttaatgttccattgaatggggcaaattattgattctaca
1981 agtgtttaattaaaaactaattgttcatcctgcagGTATTAGCGGCTACACAAATGCCTG
                                          V  L  A  A  T  Q  M  P
2041 AAGAGCCAGCTGGATGGTTCCAGGGTACAGCAGACTCTATCAGAAAATTTATCTGGGTAC
     E  E  P  A  G  W  F  Q  G  T  A  D  S  I  R  K  F  I  W  V
2101 TCGAGgtagttgatattttctcgtttatgaatgtccattcactcattcctgtagcattgt
     L  E
2161 ttctttgtaattttgagttctcctgtatttctttagGATTATTACAGTCACAAATCCATT
                                          D  Y  Y  S  H  K  S  I
2221 GACAACATTGTAATCTTGAGTGGCGATCAGCTTTATCGGATGAATTACATGGAACTTGTG
      D  N  I  V  I  L  S  G  D  Q  L  Y  R  M  N  Y  M  E  L  V
2281 CAGgtatggtgttctcttgttcctcatgtttcacgtaatgtcctgattttggattaacca
      Q
2341 actacttttggcatgcattatttccagAAACATGTCGAGGACGATGCTGATATCACTATA
                                K  H  V  E  D  D  A  D  I  T  I
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FIG. 1C

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2401 TCATGTGCTCCTGTTGATGAGAGgtaatcagttgtttatatcatcctaatatgaatatgt
      S  C  A  P  V  D  E  S
2461 catcttgttatccaacacaggatgcatatggtctaatctgctttccttttttttccccttc
2521 ggaagCCGAGCTTCTAAAAATGGGCTAGTGAAGATTGATCATACTGGACGTGTACTTCAA
           R  A  S  K  N  G  L  V  K  I  D  H  T  G  R  V  L  Q
2581 TTCTTTGAAAAACCAAAGGGTGCTGATTTGAATTCTATGgttagaaattccttgtgtaat
      F  F  E  K  P  K  G  A  D  L  N  S  M
2641 ccaattcttttgttttcctttctttcttgagatgaacccctcttttagttatttccatgg
2701 ataacctgtacttgacttattcagaaatgattttctattttgctgtagaatctgacacta
2761 aagctaatagcactgatgttgcagAGAGTTGAGACCAACTTCCTGAGCTATGCTATAGAT
                              R  V  E  T  N  F  L  S  Y  A  I  D
2821 GATGCACAGAAATATCCATACCTTGCATCAATGGGCATTTATGTCTTCAAGAAAGATGCA
      D  A  Q  K  Y  P  Y  L  A  S  M  G  I  Y  V  F  K  K  D  A
2881 CTTTTAGACCTTCTCAAgtaatcactttcctgtgacttatttctatccaactcctagttt
      L  L  D  L  L  K
2941 accttctaacagtgtcaattcttagGTCAAAATATACTCAATTACATGACTTTGGATCTG
                                 S  K  Y  T  Q  L  H  D  F  G  S
3001 AAATCCTCCCAAGAGCTGTACTAGATCATAGTGTGCAGgtaagtctgatctgtctggagt
     E  I  L  P  R  A  V  L  D  H  S  V  Q
3061 atgtgttctgtaaactgtaaattcttcatgtcaaaaagttgtttttgtttccagtttcca
3121 ctaccaatgcacgatttatgtatttcgcttccatgcatcatacatactaacaatacatt
3181 ttacgtattgtgttagGCATGCATTTTTACGGGCTATTGGGAGGATGTTGGAACAATCAA
                        A  C  I  F  T  G  Y  W  E  D  V  G  T  I  K
3241 ATCATTCTTTGATGCAAACTTGGCCCTCACTGAGCAGgtactctgtcatgtattctgtac
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FIG. 1D

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      S  F  F  D  A  N  L  A  L  T  E  Q
3301 tgcatatatattacctggaattcaatgcatagaatgtgttagaccatcttagttccatcc
3361 tgttttcttcaattagcttatcatttaatagttgttggctagaatttaaacacaaattta
3421 cctaatatgtttctctcttcagCCTTCCAAGTTTGATTTTTACGATCCAAAAACACCTTT
                           P  S  K  F  D  F  Y  D  P  K  T  P  F
3481 CTTCACTGCACCCCGATGCTTGCCTCCGACGCAATTGGACAAGTGCAAGgtatatgtctt
       F  T  A  P  R  C  L  P  P  T  Q  L  D  K  C  K
3541 actgagcacaattgttacctgagcaagattttgtgtacttgacttgttctcctccacagA
3601 TGAAATATGCATTTATCTCAGATGGTTGCTTACTGAGAGAATGCAACATCGAGCATTCTG
     M  K  Y  A  F  I  S  D  G  C  L  L  R  E  C  N  I  E  H  S
3661 TGATTGGAGTCTGCTCACGTGTCAGCTCTGGATGTGAACTCAAGgtacatactctgccaa
     V  I  G  V  C  S  R  V  S  S  G  C  E  L  K
3721 tgtatctactcttgagtataccatttcaacaccaagcatcaccaaatcacacagaacaat
3781 agcaacaaagccttttagttccaagcaatttagggtagcctagagttgaaatctaacaaa
3841 acaaaagtcaaagctctatcacgtggatagttgttttccatgcactcttatttaagctaa
3901 ttttttgggtatactacatccatttaattattgttttattgcttcttccctttgcctttc
3961 ccccattactatcgcgtcttaagatcatactacgcactagtgtctttagaggtctctggt
4021 ggacatgttcaaaccatctcaatcggtgttggacaagtttttcttgaatttgtgctacac
4081 ctaacctatcacgtatgtcatcgtttcaaactcgatccttcctgtatcatcataaatcca
4141 atgcaacatacgcatttatgcaacatttatctgttgaacatgtcatcttttgtaggtta
4201 acattatgcaccatacaatgtagcatgtctaatcatcatcctataaaatttacattttag
4261 cttatgtggtatcctcttgccacttagaacaccatatgcttgatgccatttcatccaccc
4321 tgctttgattctatggctaacatcttcattaatatcctcgcctctctgtatcattggtcc
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FIG. 1E

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4381 taaatatggaaatacattctttctgggcactacttgaccttccaaactaacgtctccttt
4441 gctcctttcttgtgtgtagtagtaccgaagtcacatctcatatattcggttttagttcta
4501 ctaagtcccgggttcgatccccctcaggggtgaatttcgggcttggtaaaaaaaatcccc
4561 tcgctgtgtcccgcccgctctcggggatcgatatcctgcgcgccaccctccggctgggca
4621 ttgcagagtgagcagttgatcggctcgttagtgatggggagcggggttcaagggttttct
4681 cggccgggaccatgtttcggtctcttaatataatgccgggagggcagtctttccctcccc
4741 ggtcgagttttagttctaccgagtctaaaaccttggactctagagtcccctgtcacaac
4801 tcacaactctagttttctatttacttctacctagcgtttattaatgatcactatatcgtc
4861 tgtaaaaagcatacaccaatgtaatccccttgtatgtcccttgtaatattatccatcaca
4921 agaaaaaaaggtaaggctcaaagttgacttttgatatagtcctattctaatcgagaagtc
4981 atctgtatcttcgtctcttgttcgaacactagtcacaaaatttttttgtacatgttcttaa
5041 tgagtccaacgtaatattccttgatattttgtcataagccctcatcaagtcaatgaaaat
5101 cacgtgtaggtccttcatttgttccttatactgctccatcacttgtctcattaagaaaat
5161 ctctctcatagttaacctttttggcatgaaacaaaatcacacagaagttgtttccttttt
5221 taagatcccacacaaaagaggtttgatctaaggaatctggatccctgacaggtttatcaa
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FIG. 1F

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FIG. 1G

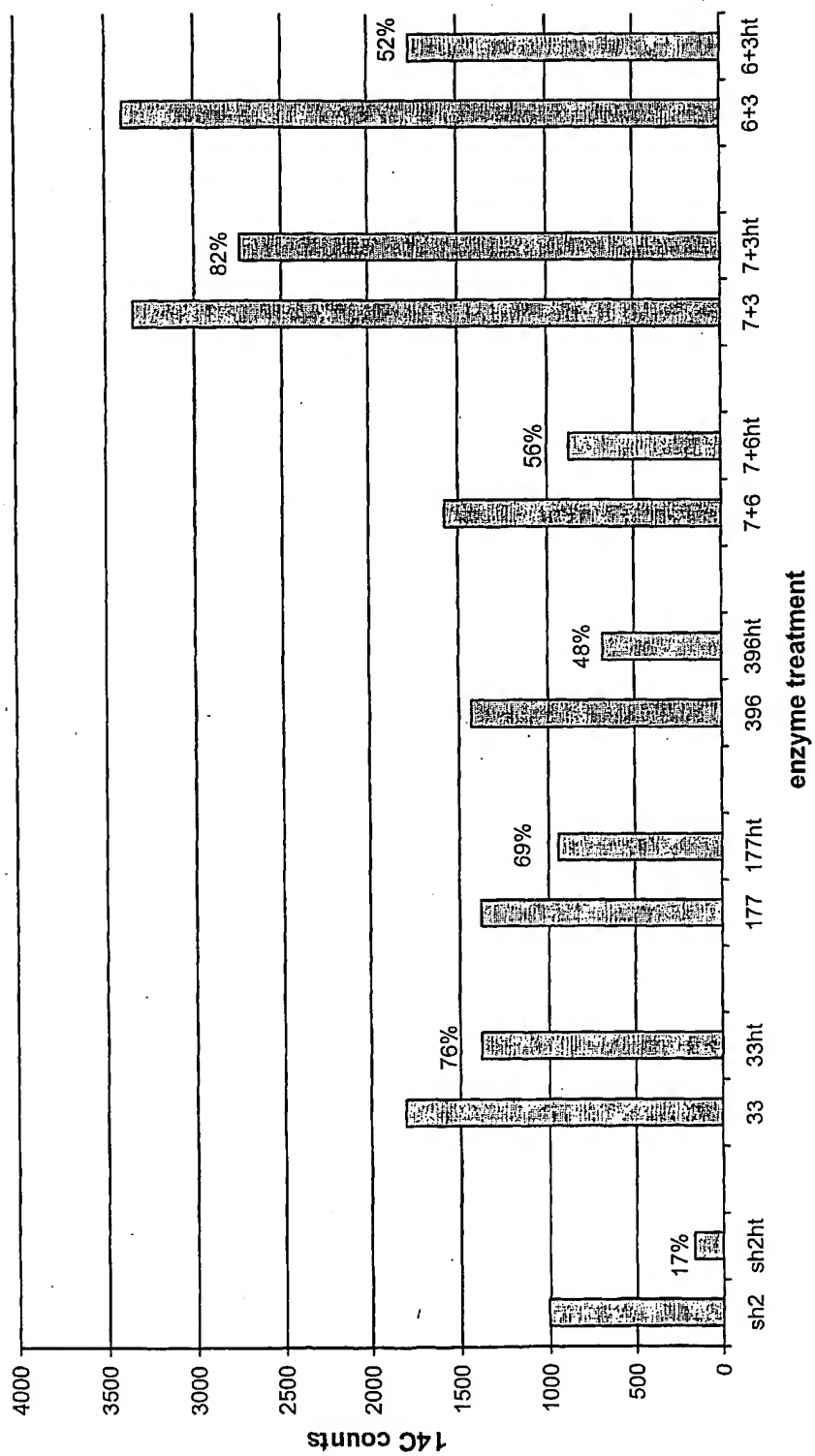
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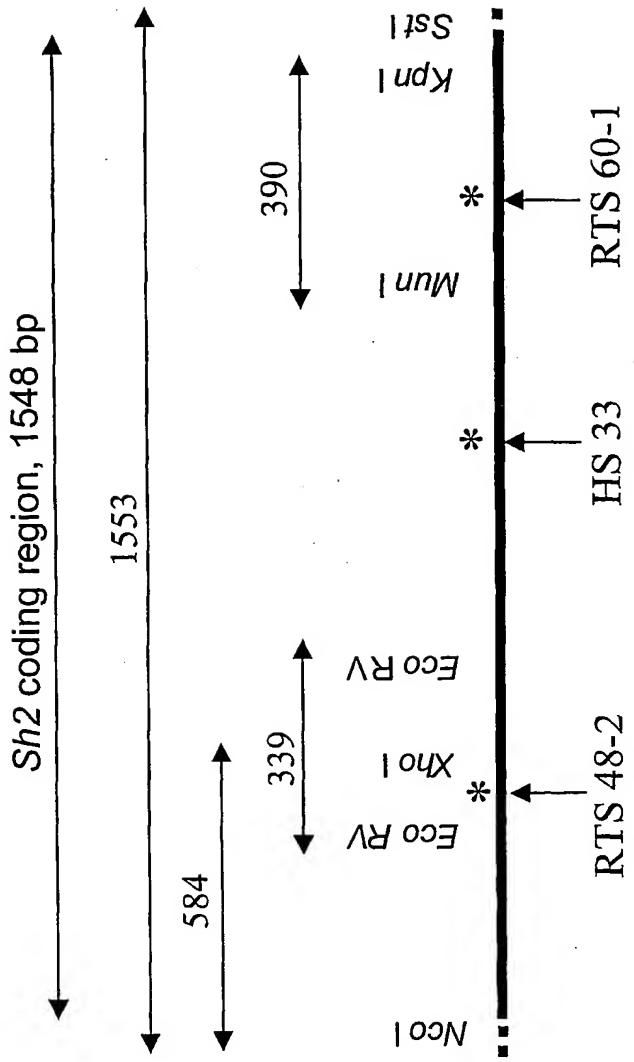
FIG. 2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RTS 48-2
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1553
Sh2 coding region, 1548 bp

FIG. 3

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Greene, Thomas W.
Burger, Brian

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